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DEP-Domain-Mediated Regulation of GPCR Signaling Responses

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SUMMARY

G protein-coupled receptors (GPCRs) mediate cellular responses to a variety of stimuli, but how specific responses are regulated has been elusive, as the types of GPCRs vastly outnumber the classes of G protein heterotrimers available to initiate downstream signaling. In our analysis of signaling proteins containing DEP domains (~90 residue sequence motifs first recognized in fly *D*ishevelled, worm *E*GL-10, and mammalian *P*leckstrin), we find that DEP domains are responsible for specific recognition of GPCRs. We examined the yeast *r*egulator of *G* protein *s*ignaling (RGS) protein Sst2 and demonstrate that the DEP domains in Sst2 mediate binding to its cognate GPCR (Ste2). DEP-domain-mediated tethering promotes downregulation by placing the RGS protein in proximity to its substrate (receptor-activated Gα subunit). Sst2 docks to the Ste2 cytosolic tail, but only its unphosphorylated state, allowing for release and recycling of this regulator upon receptor desensitization and internalization. DEP-domain-mediated targeting of effectors and regulators to specific GPCRs provides a means to dictate the nature, duration, and specificity of the response.

INTRODUCTION

DEP domains are found, often in tandem, in various proteins involved in signal transduction. This conserved sequence element was first defined (Kharrat et al., 1998) in three proteins: *D. melanogaster* Dishevelled (mammalian ortholog Dvl), an adaptor in Wingless (Wnt) signaling; EGL-10, a regulator of G protein signaling (RGS) protein that negatively regulates signaling by G protein-coupled receptors (GPCRs) in *C. elegans*; and mammalian Pleckstrin, which modulates signaling in platelets and neutrophils. In addition to pleckstrins, Dsh/Dvl, and the EGL-10-related subfamily of RGS proteins, computational analysis finds DEP domains in guanine nucleotide exchange factors (GEFs) for Rho-family GTPases of the Dbl homology class, in certain GTPase-activating proteins (GAPs), and in other signaling proteins (Burchett, 2000; Wharton, 2003). Structures for DEP domains from mouse Dvl1 (Wong et al., 2000) and Epac2 (a GEF for Rap GTPases) (Rehmann et al., 2003) and human Pleckstrin (Civera et al., 2005) reveal a conserved core comprising a three-helix bundle and a β hairpin. DEP domains have been implicated in membrane association (Pan et al., 2004; Kovoor et al., 2005), but the physiological function and mechanism of this localization have remained unresolved.

Of the five proteins in *Saccharomyces cerevisiae* with predicted DEP domains, one is Sst2, which we previously established to be the prototypic RGS protein (Dohlman and Thorner, 1997). RGS proteins attenuate signaling initiated by agonist-occupied GPCRs by stimulating hydrolysis of the GTP bound to the Gα subunit of a receptor-activated heterotrimeric G protein (Ross and Wilkie, 2000). GPCRs mediate cellular responses to a large number of stimuli, such as light, odors, tastes, peptide hormones, chemokines, and neurotransmitters (Lefkowitz, 2004). In yeast, the GPCR Ste2 initiates response to a peptide mating pheromone, α factor, by converting the associated GDP-bound Gαβγ heterotrimer (Gpa1-Ste4-Ste18) to free GTP-Gpa1 and a Ste4-Ste18 complex (Dohlman and Thorner, 2001). Sst2 squelches signaling because binding of its C-terminal RGS domain to GTP-Gpa1 stimulates nucleotide hydrolysis (Apanovitch et al., 1998; Dohlman et al., 1996), reforming GDP-Gpa1 and promoting reassembly of inactive Gαβγ.

Although the hallmark of an RGS protein is its conserved core sequence (RGS domain), which is necessary and sufficient for its GAP activity in vitro (Popov et al., 1997), we found for Sst2 that even modest N-terminal truncations (Δ1–55 and Δ1–125), far removed from its RGS domain

(420–689), totally ablated function in vivo (Dohlman et al., 1996). The N terminus of Sst2 contains a canonical DEP domain (279–358) preceded by a second DEP-like segment (50–135). Coexpression of an N-terminal fragment (1–414) and a C-terminal RGS-domain-containing fragment (415–698) complements the phenotype of *sst2*Δ cells only very weakly (Hoffman et al., 2000); hence, the N-terminal region contributes efficaciously to Sst2 function in *cis* to the RGS domain, but not in *trans*. As in other DEP-domain-containing proteins, the N terminus of Sst2 (and its worm ortholog, EGL-10) mediates membrane targeting (Hoffman et al., 2000; Koelle and Horvitz, 1996); however, use of the two-hybrid method has failed to identify a binding partner that is itself membrane localized.

Here we describe our discovery of the function of the DEP domains in Sst2. First, we show that even single point mutations in the DEP elements inactivate Sst2 function. Second, we find, conversely, that fusion of the Sst2 DEP domains to a divergent RGS domain confers the ability to act in the pheromone response pathway. Third, using a loss-of-function point mutation in an unbiased selection for dosage suppressors, we identify the physiologically relevant interaction partner. Fourth, we confirm using biochemical methods and cell imaging methods that the DEP domains are necessary and sufficient for Sst2 binding to this target. Finally, we show how this interaction contributes to desensitization and provide a mechanism for its regulation.

RESULTS

DEP Domains Are Essential for Sst2 Function

The N-terminal portion of Sst2 contains a degenerate (DEP-A) and a more canonical (DEP-B) DEP domain (Figure 1A). In DEP structures from Dvl1, Epac2, and Pleckstrin 1, an upstream helix (α1) sandwiches a β hairpin (β1-β2) between two downstream helices (α2 and α3). To test whether either Sst2 DEP element is essential for its function, portions of predicted helical segments in DEP-A and DEP-B, and all of DEP-B, were deleted. Pheromone-initiated signaling blocks cell-cycle progression in the G1 phase, which can be monitored by a halo of growth inhibition in a lawn of cells. Because Sst2 reverses G protein activation, it promotes recovery from this growth arrest. When Sst2 is present, even a high dose of pheromone elicits only a small halo, whereas in an *sst2*Δ null mutant, even a ten-fold lower dose produces a very large halo (Figure 1B, left). All of the DEP-domain deletions, even overexpressed from the strong *GAL1* promoter, as judged by immunoblotting (data not shown), caused a phenotype as severe as the complete absence of Sst2 (Figure 1B, right). For less drastic perturbation, we introduced double Pro substitutions into predicted α helices in DEP-A and DEP-B and into the spacer between them and tested them in the same way. These changes in DEP-A or DEP-B also caused a defect as severe as an *sst2*Δ mutation, whereas alteration of the intervening region had a milder effect (Figure 1C). Thus, both DEP elements in Sst2 are as important for its cellular function as its RGS domain. Moreover, these data indicate that DEP-A and DEP-B work cooperatively because alteration of either was sufficient to cripple Sst2 function.

DEP Domains of Sst2 Direct Its RGS Activity to the Pheromone Response Pathway

If the DEP domains in Sst2 target this regulator to its site of action, we reasoned that attachment to another RGS domain that normally does not function in pheromone response might permit it to act on Gpa1-GTP. For this purpose, we selected yeast Rgs2, which acts on the Gα subunit (Gpa2) activated by a different GPCR (Gpr1) in a glucose-sensing pathway (Versele et al., 1999). Indeed, we found that, unlike overexpression of the DEP-domain-containing fragment of Sst2 alone or the RGS-domain-containing fragment of Rgs2 alone, the corresponding Sst2-Rgs2 chimera was nearly as potent as native Sst2 in promoting recovery from pheromone-imposed growth arrest, regardless of the strain background (Figure 2A).

Signal dampening by Sst2 also squelches expression of a pheromone-inducible reporter gene, *FUS1*^{prom}*-lacZ* (Hoffman et al., 2002). This method was used to test efficacy of a more extensive set of Sst2-Rgs2 chimeras (Figure 2B, upper left) in *sst2*Δ cells. If the specificity for Gpa1 resided in those portions of the Sst2 RGS domain that are most divergent from those in the Rgs2 RGS domain, then swapping those segments from Sst2 into Rgs2 might confer the desired specificity, which it did not (Figure 2B). In contrast, attachment of the N-terminal DEP-domain-containing segment of Sst2 to the RGS domain of Rgs2 (with or without its C-terminal extension) squelched pheromone-induced reporter-gene expression even more potently than wild-type Sst2 did (Figure 2B, lower right), whereas the DEP domain of Sst2 alone was previously shown to be ineffective in this same assay (Hoffman et al., 2000). Thus, the specificity for RGS action against Gpa1 resides primarily in the DEP-domain-containing segment of Sst2.

Signaling by heterotrimeric G proteins starts at the plasma membrane where GPCRs reside. Given the evidence that other DEP domains mediate membrane association, they could represent a novel lipid-binding motif that confers specificity simply by localizing Sst2 to the membrane bilayer. Using surface plasmon resonance, we found that the Sst2 N-terminal segment, but not its RGS domain, has a propensity to interact with phospholipids. This affinity for phospholipids is rather nonspecific in comparison, for example, to the preference of a bona fide PH domain for PtdIns4,5P_2 (see Figure S1A in the Supplemental Data available with this article online). Moreover, fusion of a known membrane-targeting motif (N-myristoylated and S-palmitoylated N-terminal ten residues of Gpa1) to DEP-domain-defective *sst2* mutants (see below) did not fully restore function (Figure S1B). Hence, although lipid-binding propensity could contribute to DEP function,

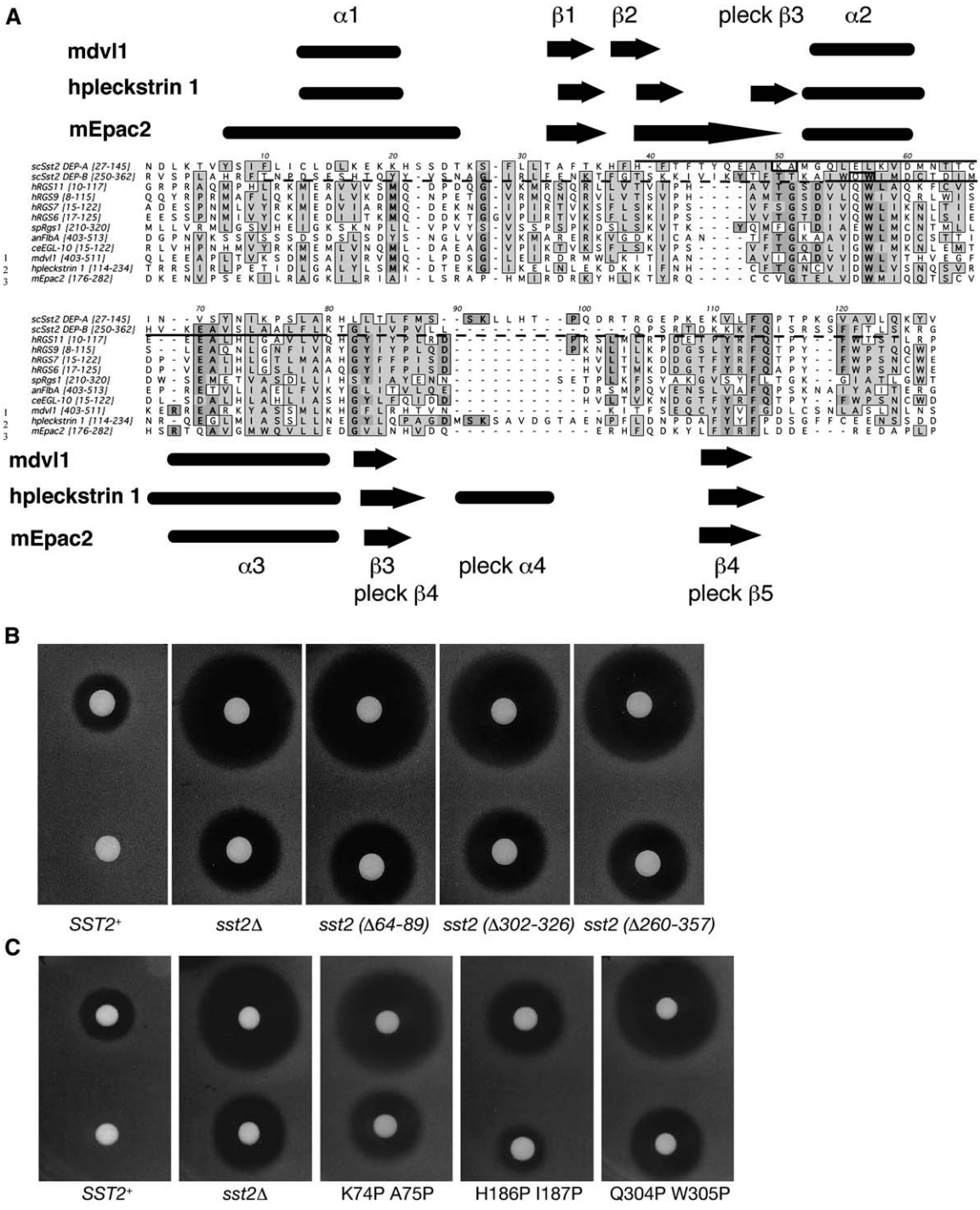

Figure 1. DEP Domains in Sst2 Are Essential for Its Function

(A) The degenerate DEP-A and canonical DEP-B domains in Sst2 are aligned with ten others; three (1–3) have solved structures, whose secondary structure elements are depicted.

(B) An *sst2*Δ mutant (YDB100) was transformed with a low-copy (*CEN*) vector or the same vector expressing wild-type *SST2* from the *GAL1* promoter or the indicated internal deletions (over- or underlined in [A]). Representative transformants were tested for sensitivity to pheromone-induced growth arrest using an agar diffusion (halo) bioassay.

(C) The indicated double-Pro substitutions (boxed in [A]) were also tested, as in (B).

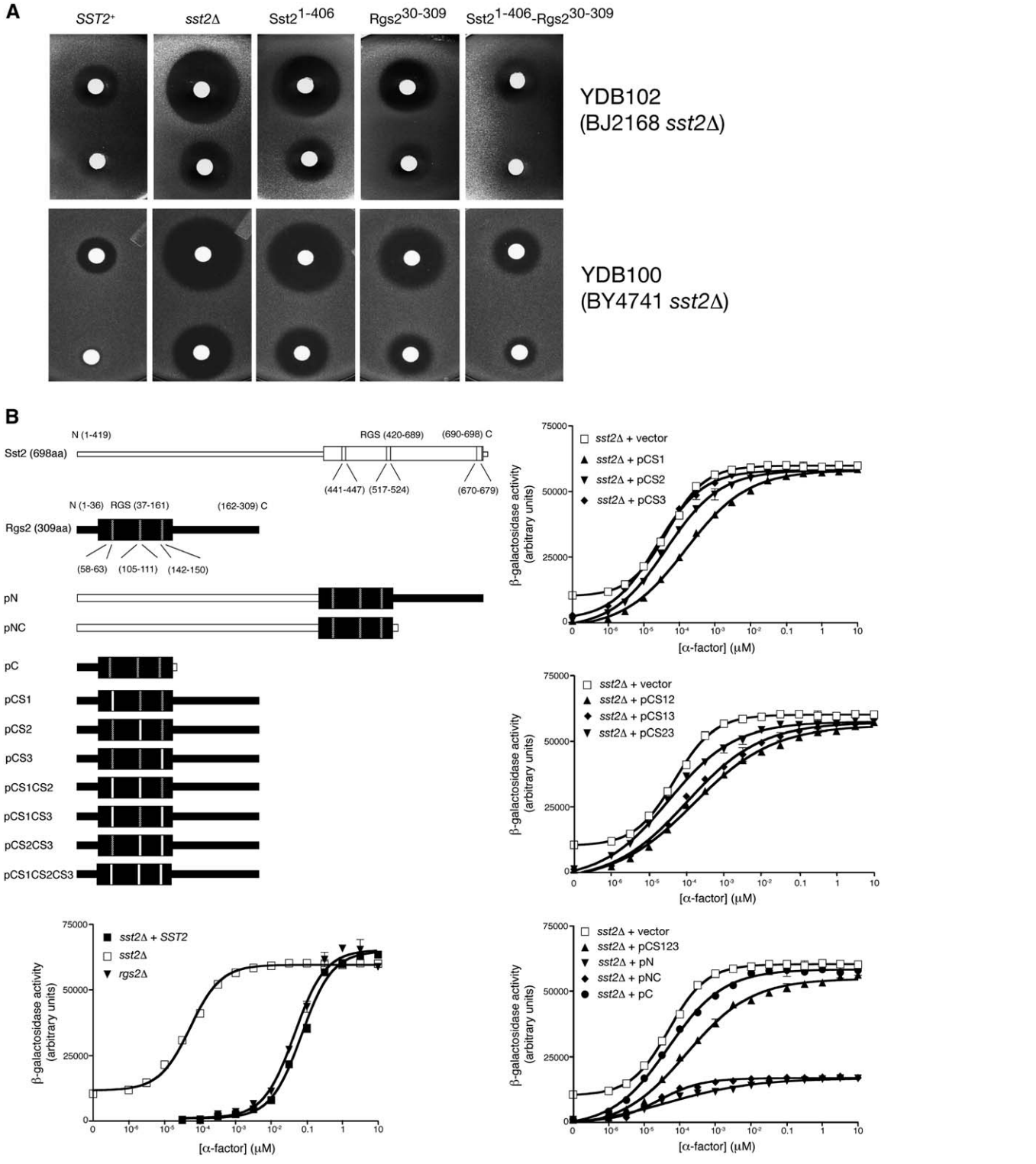

Figure 2. DEP Domains of Sst2 Confer Specificity for the Pheromone Response Pathway

(A) Ability of Sst2(1–406), Rgs2(30–309), or an Sst2(1–406)-Rgs2(30–309) chimera to complement an *sst2*Δ mutation was tested in two strain backgrounds as in Figure 1B. The first 30 residues of Rgs2 were not included because they may exert an autoinhibitory effect on the RGS domain (M. Versele, personal communication).

it seemed to us that the DEP domains of Sst2 might also mediate interaction with a specific protein target.

Positive Selection for Loss-of-Function Alleles Identifies a Critical DEP-Domain Residue

To implement a genetic approach for identifying potential interaction partners, we first generated single point mutations in the DEP-domain-containing region of Sst2 that disrupt its function, but not its expression or stability. To do so, we used error-prone PCR to generate an unbiased library of DNA fragments mutagenized exclusively in the DEP-domain region that were reintegrated into the *SST2* coding sequence via in vivo repair of a suitably gapped recipient plasmid (Figure S2A). We then devised and applied a positive selection to identify loss-of-function alleles, and candidates that expressed full-length Sst2 at a normal level were identified, sequenced, and characterized as described in Experimental Procedures. Two independently isolated mutants carried different base pair changes altering the same residue (Q304E and Q304L), and both resulted in complete loss of function. Modeling of Sst2 DEP-B on structures of the three known DEP domains predicts that Q304 is solvent exposed. It has been proposed that the corresponding residue in mouse Dsh participates in a dipole that is a good candidate for a protein-protein interaction interface (Wong et al., 2000). Mutagenesis of the equivalent residue in fly Dsh disrupts Wnt signaling (Penton et al., 2002). We verified our results by introducing the same substitutions, as well as Q304N, into otherwise pristine *SST2* DNA using site-directed mutagenesis. When expressed at an endogenous level, even the conservative substitutions (Q304N and Q304E) displayed nearly total loss of function (Figure S2B), confirming that Q304 is critical. Q304N and Q304E coimmunoprecipitate with Gpa1 as well as wild-type Sst2 (data not shown). Importantly, we noted that, when highly overexpressed (from a high-copy plasmid and driven by the strong inducible *GAL1* promoter), Q304E and especially Q304N displayed significant residual function (Figure S2C). This latter observation suggested that we might be able to identify the protein target that interacts with the DEP domain by screening for dosage suppressors that restore Sst2 function when the Q304N mutant is expressed at its normal level.

Positive Selection for Suppressors of Sst2(Q304N) Identifies the GPCR Ste2

We reasoned that the defect in an Sst2 mutant specifically crippled in its DEP domains, like the Q304N allele, might be rescued, at least partially, if we overexpressed its binding partner from a multicopy plasmid. In this way, from a library of such overexpressed genes, we might identify in an unbiased way the protein that normally associates with Sst2 via its DEP domains. Hence, we developed conditions to positively select for restoration of Sst2(Q304N) function (basically, ability to grow on an α factor concentration that blocks growth of the Sst2(Q304N) mutant carrying empty library vector) and conducted a genome-wide selection for dosage suppressors. Candidate colonies were screened secondarily for those that only grew upon exposure to pheromone in the presence of Sst2(Q304N), but not in its absence. From $\geq$20,000 initial transformants, 30 grew in the presence of pheromone, but just 6 permitted growth only when Sst2(Q304N) was present. Upon retesting, only one of the six reproducibly permitted growth in an Sst2(Q304N)-dependent manner. The plasmid rescued from this isolate contained six open reading frames; one was the *STE2* gene, which encodes the α factor receptor. To verify that *STE2* was responsible for the suppression observed, an independently constructed plasmid expressing only the *STE2* gene from the strong constitutive *TDH3* promoter (David et al., 1997) was tested. As judged by both the growth assay (data not shown) and the halo assay (Figure 3A), overexpressed Ste2 had no effect on pheromone sensitivity of *sst2*Δ cells, but, like the library isolate, it significantly reduced pheromone sensitivity of cells expressing Sst2(Q304N). As judged by immunoblotting (Figure 3B), this effect could not be attributed to any elevation in the level of the mutant Sst2 protein in the absence or presence of α factor. (*SST2* is a pheromone-inducible gene [Dohlman et al., 1996].) As expected for rescue arising from authentic protein-protein interaction, suppression was allele specific. Ste2 overexpression ameliorated significantly only the pheromone sensitivity of cells expressing Sst2(Q304N) (the allele with the greatest residual function) and not Sst2(Q304E) or Sst2(Q304L) (data not shown).

Ste2 Physically Associates with Sst2 via Its DEP Domains

We found, first, that endogenous Ste2 detergent solubilized from purified plasma membranes, regardless of its state of N-glycosylation, binds to c-Myc epitope-tagged Sst2 immobilized on beads using anti-myc mAb 9E10 (Figure 4A). Interaction was specific because no bound species were observed if beads lacked Sst2 or if membrane extracts were prepared from *ste2*Δ cells. Most importantly, no Ste2 was retained when beads were coated with an equivalent amount of Sst2(Q304N) (Figure 4A). No detectable Gpa1 was present in the pull-down, as judged by immunoblotting with an anti-Gpa1 antibody (Dohlman et al., 1993). We found, second, that His_6-Sst2 (produced by baculovirus expression and purified by FPLC) bound to FLAG-tagged Ste2 (detergent solubilized from plasma membranes of *sst2*Δ *ste2*Δ *gpa1*Δ *ste4*Δ cells) immobilized on beads with anti-FLAG epitope mAb (Figure 4B). Taken together, these in vitro data show that Sst2 binds directly to Ste2 in a DEP-dependent (but not Gpa1-dependent) manner.

(B) Sst2 or the indicated Sst2-Rgs2 chimeras (upper left), expressed from the *ADH1* promoter on a *CEN* plasmid in *sst2*Δ cells, were tested for their efficacy in squelching pheromone-induced expression of a reporter gene (remaining panels). Data shown are representative of three independent experiments. Values given are averages of samples (assayed in triplicate) for a typical experiment; error bars indicate the standard error of the mean (SEM).

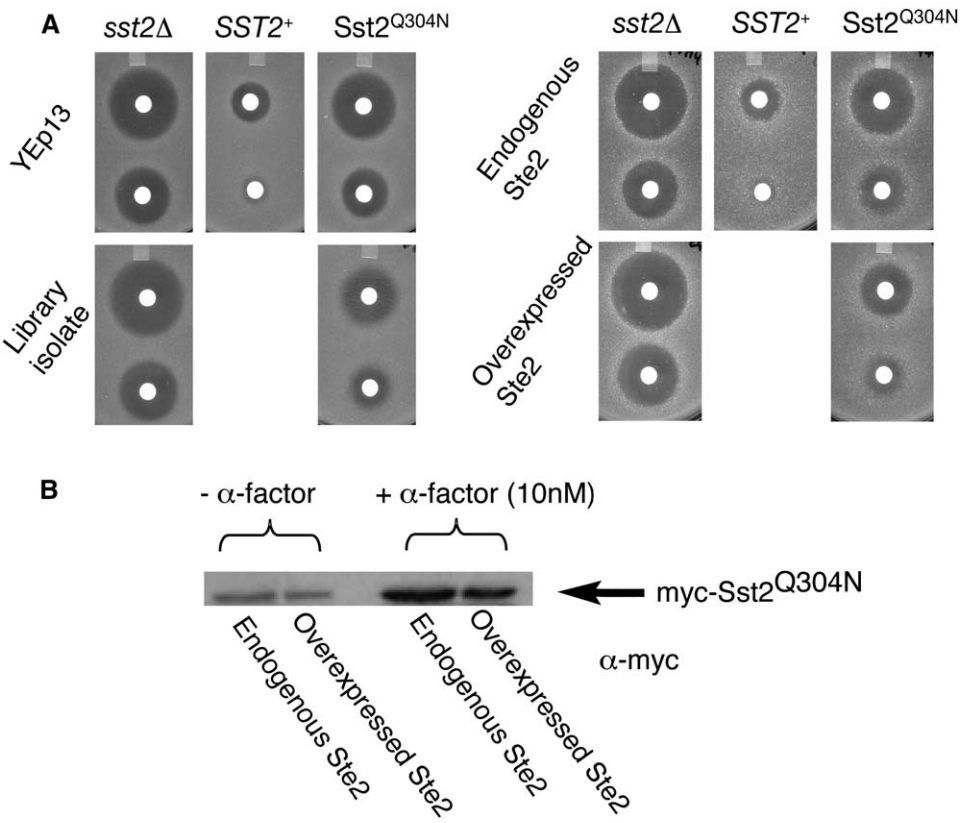

Figure 3. Overexpression of Ste2 Ameliorates the Defect of an Sst2 DEP-Domain Mutant

(A) YDB100 (*sst2*Δ *leu2*Δ *ura3*Δ) (left panels) carrying an empty *URA3*-marked *CEN* vector (left column) or the same vector expressing myc-Sst2(Q304N) (right column) was transformed with empty *LEU2*-marked library vector (YEp13) (top row) or the dosage suppressor isolated from a genomic library in the same vector (bottom row). YDM400 (*sst2*Δ *trp1*Δ *ura3*Δ) (right panels) carrying the same *CEN* vector lacking (left column) or expressing myc-Sst2(Q304N) (right column) was transformed with empty *TRP1*-marked vector (YEplac112) (top row) or a *TRP1*-marked multicopy (2 μm DNA) plasmid expressing *STE2* from the strong constitutive *TDH3* promoter (bottom row). For comparison, YDB100 and YDM400 were transformed with a *URA3*-marked *CEN* vector expressing wild-type myc-Sst2 (center, left, and right panels). All were tested for pheromone sensitivity as in Figure 1B.

(B) Cells expressing myc-Sst2(Q304N) with or without overexpressed Ste2 in either the absence or presence of pheromone were lysed, resolved by SDS-PAGE, and analyzed by immunoblotting with anti-c-Myc mAb 9E10.

To corroborate this conclusion in a physiological setting, we used the split-ubiquitin two-hybrid method that was developed to assess interaction of membrane-associated proteins (Fetchko and Stagljar, 2004). One protein is fused to an N-terminal (N-UbG) fragment defective for association with a C-terminal (C-Ub) fragment that is fused to the other protein (Figure 4C). Interaction between the proteins forces ubiquitin reassembly. If reassembled, ubiquitin C-terminal hydrolases clip off a transcription factor (LexA-VP16) fused to the C terminus of C-Ub. Once freed from its membrane tether, LexA-VP16 enters the nucleus and induces expression of a *lacZ* reporter gene with LexA binding sites. Our tester cells all expressed an Ste2-C-Ub-LV fusion and coexpressed association-defective N-UbG alone, wild-type Sst2-N-UbG, or each Sst2 DEP-domain mutant fused to N-UbG. Every transformant expressing Sst2-N-UbG and Ste2-C-Ub-LV showed robust reporter-gene expression at a level equivalent to that of the positive control (Figure 4D). For this control, cells were cotransformed with an Ste2-N-UbI chimera that binds Ste2-C-Ub-LV for two reasons: N-UbI interacts constitutively with C-Ub, and Ste2 self-associates to form dimers (Overton et al., 2003). Most importantly, like the negative control expressing N-UbG alone, no reporter expression was seen when Sst2-N-UbG carried any Q304 mutation (Figure 4D). As a specificity control, two other GPCR-C-Ub-LV fusions were tested for interaction with Sst2-N-UbG, V2 vasopressin receptor, and calcitonin receptor. These receptor-C-Ub-LV chimeras displayed a somewhat higher background than Ste2-C-Ub-LV alone did; however, unlike Ste2-C-Ub-LV, neither showed any enhancement of reporter-gene expression in the presence of Sst2-N-UbG or any of the DEP-domain mutants (data not shown). Thus, interaction of Sst2 with Ste2 was only observed with this specific GPCR-RGS protein pair and was DEP domain dependent.

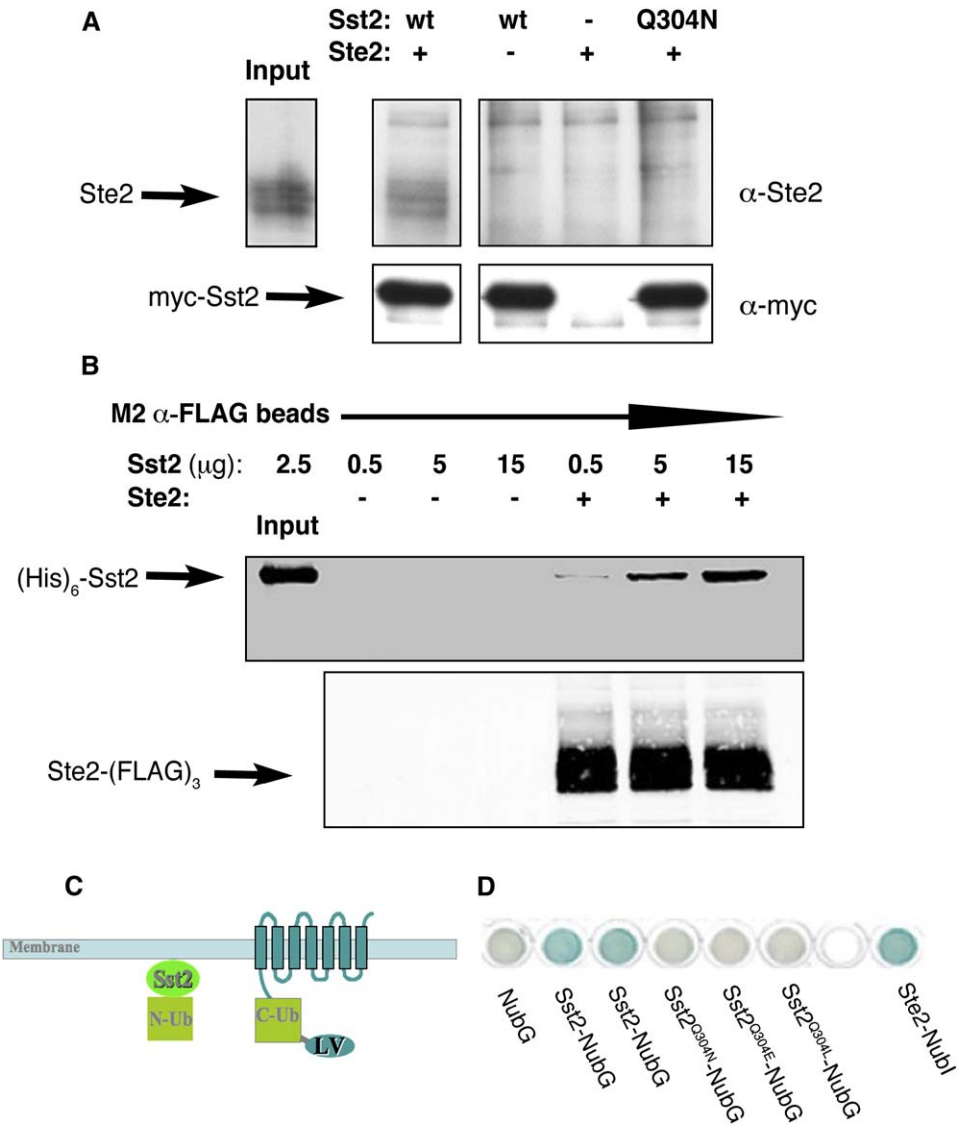

Figure 4. Sst2 Binds Ste2 via Its DEP Domain Both In Vitro and In Vivo

(A) Ste2 solubilized with 0.5% n-dodecyl-beta-D-maltopyranoside (DoBM; Calbiochem, San Diego, CA, USA) from plasma membranes isolated by sucrose-gradient flotation from YDB100 (*sst2*Δ) (+) (input, 5% of total added) or a control from YDB105 (*sst2*Δ *ste2*Δ) (–) was incubated with anti-c-Myc antibody-decorated Protein A/G beads coated with myc-Sst2(WT) or myc-Sst2(Q304N) expressed from the *GAL1* promoter on a *CEN* vector in YDB104 (*sst2*Δ *ste2*Δ *ste4*Δ *gpa1*Δ), a derivative of a protease-deficient strain (BJ2168), or with an identical extract of YDB104 expressing empty vector (–). After washing, bound proteins eluted in 5% SDS sample buffer at 37°C and resolved by SDS-PAGE were analyzed by immunoblotting with rabbit polyclonal anti-Ste2 antibodies (upper panel) or mouse anti-c-Myc mAb 9E10 (bottom panel). A representative experiment (n = 3) is shown in which the indicated controls were run in parallel; to permit side-by-side comparison, the input and one other lane were moved from their original position in the gel and are therefore in separate boxes.

(B) The indicated amounts of His_6-Sst2, produced by baculovius expression and purified by FPLC, were incubated with anti-FLAG mAb M2-decorated beads or the same beads coated with a DoBM extract of plasma membranes from YDB118 (*ste2*Δ *sst2*Δ *gpa1*Δ *ste4*Δ) expressing pYES-STE2-FLAG. After washing and elution with FLAG peptide, released proteins were analyzed by immunoblotting with anti-His_6 (QIAGEN) and a rabbit polyclonal anti-FLAG IgG (Sigma).

(C) Split-ubiquitin two-hybrid method (Fetchko and Stagljar, 2004) to assess the ability of fusions of Sst2, Sst2(Q304N), Sst2(Q304E), and Sst2(Q304L) to N-UbG to interact with Ste2-C-Ub-LV.

(D) As a negative control (left), the ability of N-UbG alone to interact with Ste2-C-Ub-LV was tested. As a positive control (right), the ability of Ste2-N-UbI to interact with Ste2-C-Ub-LV was tested. N-UbI versus N-UbG refers to an I13G mutation, which prevents spontaneous association of N-Ub and C-Ub.

Sst2 Binds via Its DEP Domains to the Cytosolic Tail of Ste2

To demonstrate that Sst2 and Ste2 interact in vivo and follow the dynamics of their association in real time in live cells, we constructed a fully functional fusion of Sst2 to GFP. For expression at its endogenous level, this construct was integrated into the *SST2* chromosomal locus in *MAT***a** cells, and its subcellular localization was examined using confocal fluorescence microscopy. To rule out any contribution of the interaction of the Sst2 RGS domain with its GTP-bound Gα substrate (Gpa1), the *GPA1* locus was replaced with *GPA1(G302S)*, a mutation that abrogates Gpa1 binding to the Sst2 RGS domain but does not otherwise perturb its function (DiBello et al., 1998). When exposed to α factor, cells underwent the expected pheromone-induced morphogenesis and formed the characteristic pear shape ("shmoo"). Concomitantly, Sst2-GFP was recruited prominently to the tip of the shmoo (Figure 5A), where it has been amply demonstrated previously that newly made Ste2 and other integral membrane proteins are inserted (Heiman and Walter, 2000; Dohlman and Thorner, 2001). Under identical conditions, Sst2(Q304N)-GFP was not recruited to the shmoo tip, indicating that intact DEP domains are required for binding of Sst2 to Ste2. Consistent with this conclusion, wild-type Sst2-GFP was never observed at the tips of the shmoos that form spontaneously at a high frequency in *ste2*Δ cells (Figure 5A). (Such morphogenesis occurs because absence of receptor destabilizes the associated Gαβγ heterotrimer, causing stochastic firing of the pheromone response pathway [Reneke et al., 1988; Siekhaus and Drubin, 2003].)

To demonstrate colocalization of Sst2 and Ste2 directly and the role of the DEP domains in their association, Sst2-GFP was introduced into strains in which chromosomally expressed Ste2 was fused to mCherry, a variant of mRFP (Shaner et al., 2004). In otherwise wild-type cells and even in the absence of pheromone, Ste2-mCherry undergoes robust constitutive ubiquitin-dependent endocytosis (Hicke et al., 1998; Katzmann et al., 2004). Thus, as expected, the bulk of the red fluorescence resides in the lysosome-like vacuole. We also noted prominent green fluorescence in the perivacuolar region, as expected if at least some of the Sst2 is internalized from the cytosolic face of the plasma membrane along with Ste2 (Figure 5B, top panels). To prevent constitutive endocytosis, seven Lys residues in the cytosolic tail of Ste2-mCherry that promote endocytosis were mutated to Arg (Terrell et al., 1998). Indeed, as expected, Ste2(7K-to-R)-mCherry displayed prominent decoration of the plasma membrane (Figure 5B, lower panels). Most strikingly, the Sst2-GFP signal now was also most pronounced at the plasma membrane (Figure 5B, lower panels). Thus, localization of Sst2-GFP tracked with localization of Ste2-mCherry (Figure S3), as expected for two proteins that associate rather stably and with high affinity. When identical experiments were conducted using Sst2(Q304N)-GFP (Figure 5C), green fluorescence remained exclusively in the cytosol, even in the cells expressing Ste2(7K-to-R)-mCherry (Figure 5C, lower panels). Thus, colocalization of Sst2 with Ste2 requires the function of its DEP domains.

We demonstrated before that deleting the C-terminal cytosolic tail (residues 297–431) of Ste2 prevents its endocytosis and also that such a C-terminally truncated receptor confers an elevated level of sensitivity to pheromone, comparable to that of an *sst2*Δ mutation (Konopka et al., 1988; Reneke et al., 1988). We found that an mCherry derivative of tailless Ste2 was still located in the plasma membrane, as expected, whereas Sst2-GFP in the same cells was located exclusively in the cytosol (Figure 6A), contrary to its clear plasma membrane association in cells expressing mCherry-tagged full-length Ste2(7K-to-R). Hence, the cytosolic tail of Ste2 is necessary for recruitment of Sst2 in vivo. The Ste2 tail is also sufficient to bind Sst2. In cells lacking native Ste2, *GAL1*-promoter-expressed Ste2(297–431)-mCherry, tethered to the plasma membrane via its fusion to Gpa1(1–10), recruited endogenously expressed Sst2-GFP to the cell membrane, especially in the bud (Figure 6B, upper panels). Although expression of Sst2-GFP is drastically reduced in the absence of basal G protein-dependent signaling (Roberts et al., 2000), even with a constitutively active MAPKKK (*STE11-4* allele) present, production of the tail chimera brings some Sst2-GFP to the membrane in cells lacking both Ste2 and Gpa1 (Figure 6B, lower panels).

To examine the generality of these findings for another GPCR, we tested whether Ste3 (the **a** factor receptor of *MAT*α cells) associates with Sst2 (because an *sst2*Δ mutation makes *MAT*α cells hypersensitive to **a** factor). As observed for Ste2 (Figure 5B), preventing ubiquitylation and endocytosis of Ste3 with 3K-to-R mutations greatly increased the amount of Sst2 recruited to the plasma membrane (Figure S4). The C-terminal tails of Ste2 and Ste3 bear little discernible sequence identity, suggesting that the element recognized by Sst2 in each receptor is not a simple sequence motif.

Phosphorylation of the Cytosolic Tail of Ste2 Displaces Sst2

We noted that, in naive cells, both endocytosis-defective Ste2(7K-to-R)-mCherry and Sst2-GFP colocalize at the plasma membrane all around the perimeter of the cell (Figure 5B and Figure 6A). In contrast, by the time cells form prominent shmoos after exposure to pheromone, the Sst2-GFP concentrates at the tip of the shmoo and does not associate with the Ste2(7K-to-R)-mCherry elsewhere in the plasma membrane (Figure 6C, upper panels). Prior work has shown that pheromone binding to receptor promotes phosphorylation of its cytosolic tail by two membrane-anchored casein kinase I isoforms (Yck1 and Yck2) (Feng and Davis, 2000) as a prerequisite to enhanced ubiquitylation and endocytosis (Chen and Konopka, 1996; Hicke et al., 1998). Because Ste2(7K-to-R)-mCherry cannot undergo endocytosis, we presumed that, once phosphorylated, this receptor remains in the membrane but is displaced from the shmoo tip by newly incorporated, unliganded receptor, which has the least

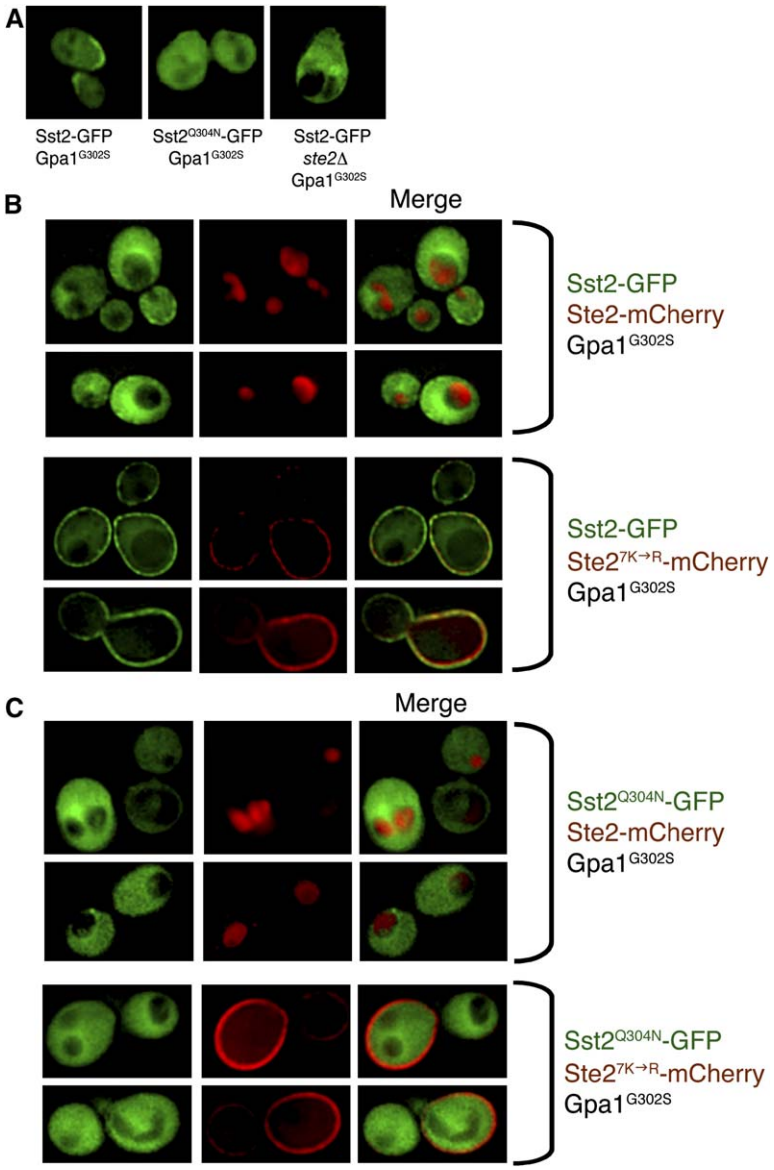

Figure 5. Sst2 Colocalizes with Ste2

(A) An exponentially growing culture of a Gpa1(G302S) mutant (which abrogates binding of the Sst2 RGS domain) with Ste2 or without (*ste2*Δ) and expressing Sst2-GFP or Sst2(Q304N)-GFP from the *SST2* promoter at its chromosomal locus was treated with 500 nM α factor for 1 hr and examined by confocal fluorescence microscopy.

(B) An exponentially growing culture of a Gpa1(G302S) mutant expressing Sst2-GFP as in (A) and also coexpressing from the *STE2* promoter at its chromosomal locus either Ste2-mCherry (upper two rows) or endocytosis-defective Ste2(7K-to-R)-mCherry (bottom two rows) was examined as in (A), but in the absence of any pheromone treatment.

(C) As in (B), except that the cells expressed Sst2(Q304N)-GFP. Representative cells are shown in all panels.

opportunity to undergo any posttranslational modification. Hence, we reasoned that phosphorylation of the Ste2 cytosolic tail might prevent Sst2 docking and thus be responsible for the pattern observed. Indeed, in cells exposed to pheromone, but in which Yck1- and Yck2-mediated phosphorylation was ablated by appropriate mutations, Sst2-GFP associated with Ste2(7K-to-R)-mCherry around the entire perimeter of the cells (Figure 6C, middle panels). To demonstrate that the relevant target of Yck1 and Yck2 that prevents Sst2 interaction is the cytosolic tail of Ste2 itself, rather than any other potential substrate, we mutated all 19 of the Ser and Thr residues in the region of the tail that has been delimited by prior work as containing the physiologically relevant phosphorylation sites (Chen and Konopka, 1996; Hicke et al., 1998). In the cells expressing Ste2(7K-to-R, 19S/T-to-A)-mCherry, the Sst2-GFP signal was now congruent with the red fluorescence (Figure 6C, bottom panels) in every cell, unlike cells expressing Ste2(7K-to-R)-mCherry, where the Sst2-GFP is located exclusively at the shmoo tip (Figure 6C, upper panels). Thus, agonist-stimulated phosphorylation of the Ste2 cytosolic tail is responsible for dissociating Sst2 prior to the ligand-induced endocytosis of this receptor.

DISCUSSION

We used experimental advantages of yeast cells to discern the function of DEP domains in the prototypic RGS protein Sst2. Given that DEP domains are found in proteins that participate in signaling, and given how extensively studied signaling pathways are, it is surprising that a physiological role for DEP domains had not previously

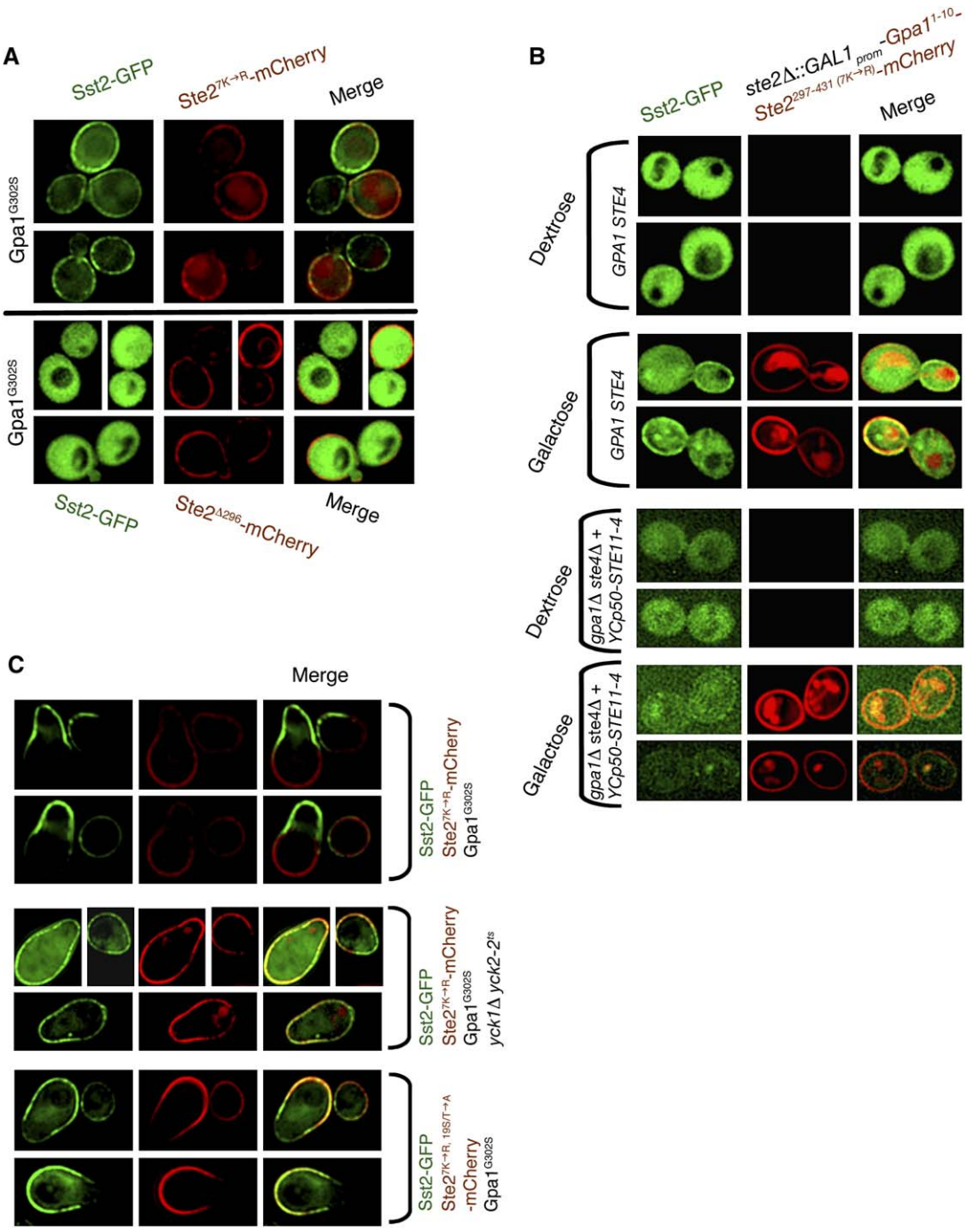

Figure 6. Sst2 Associates with the Unphosphorylated State of the Cytosolic Tail of Ste2

(A) An exponentially growing culture of a Gpa1(G302S) mutant expressing Sst2-GFP as in Figure 5 and also coexpressing from the *STE2* promoter at its chromosomal locus either endocytosis-defective Ste2(7K-to-R)-mCherry (upper two rows) or endocytosis-defective Ste2(Δ296)-mCherry (bottom two rows) was examined as in Figure 5.

(B) Otherwise wild-type cells (YDB120) expressing Sst2-GFP as in Figure 5 and also coexpressing from the *GAL1* promoter at the *STE2* locus a Gpa1(1–10)-Ste2(297–431)-mCherry chimera were grown on glucose (upper panels) or induced with galactose (lower panels), and examined as in (A). YDB122 (*gpa1*Δ *ste4*Δ [YCp50-*STE11-4*]) expressing the Gpa1(1–10)-Ste2(297–431)-mCherry chimera and weakly expressing Sst2-GFP was analyzed as in (A).

(C) An exponentially growing culture of a Gpa1(G302S) mutant expressing Sst2-GFP and endocytosis-defective Ste2(7K-to-R)-mCherry (upper panels) or carrying the *yck1*Δ *yck2*^{ts} mutations (middle panels) was shifted to restrictive temperature (37°C), treated with 500 nM α factor for 1 hr,

been defined. We demonstrated, first, that the DEP domains are as necessary for Sst2 function as its hallmark RGS domain. We then showed that the Sst2 DEP domains are sufficient to confer on a distantly related (40% similarity) RGS domain efficient action on Gpa1-GTP. By contrast, membrane targeting of the Sst2 RGS domain via other means only inefficiently complemented an *sst2*Δ mutation. Likewise, mammalian RGS4 (a small "RGS-domain-only" protein) was first isolated by its ability to reduce pheromone hypersensitivity of *sst2*Δ cells (Druey et al., 1996), and this weak rescue required the N-terminal, basic, amphipathic membrane-binding α-helix of RGS4 (Srinivasa et al., 1998). These differences in potency suggested to us that the DEP domains of Sst2 must do more than just tether this RGS protein to the cell membrane.

Nonetheless, our surface plasmon resonance results showed that the Sst2 DEP domains have phospholipid-binding propensity in vitro, consistent with the membrane binding reported for other DEP domains in vivo. Basic residues cluster on a surface of the DEP domain of mouse Dvl1 distinct from its proposed protein-protein interaction interface (Wong et al., 2000). Modeling indicates that the Sst2 DEP domains also have a basic surface, which may mediate binding to phosphates in the head groups of phospholipids, explaining its observed affinity for membranes (Dohlman et al., 1996).

Because its DEP domains are necessary and sufficient to direct Sst2 selectively to the pheromone response pathway, it seemed likely that these domains also interact with a specific protein target in that pathway. To use genetic means to identify that component, we identified conservative substitutions at a single residue (Q304) in DEP-B that severely crippled Sst2 but retained residual function (revealed when the protein was markedly overexpressed). Based on the fact that complex formation between a mutant protein and its normal binding partner can often be restored through mass action by greatly elevating expression of that partner, we then selected for dosage suppressors of the mutant Sst2(Q304N) using a genomic DNA library expressed at a high level from a multicopy plasmid. This approach yielded one candidate, the α factor receptor (Ste2).

We confirmed the authenticity of this finding in multiple ways. First, we demonstrated that detergent-solubilized Ste2 binds in vitro to immobilized Sst2 and that Q304N eliminates that binding. No Gpa1 was detectable in these pull-downs. Second, we found that purified recombinant Sst2 bound to immobilized Ste2 immunopurified from cells lacking Gpa1. Thus, Sst2 and Ste2 bind directly. Third, using the split-ubiquitin two-hybrid method, we showed that Sst2 and Ste2 associate in vivo and that mutations of Q304 prevented this interaction. Fourth, we demonstrated using differentially tagged fluorescent derivatives of Ste2 and Sst2 that this regulator (but not the Q304N mutant) colocalizes with its cognate receptor. Moreover, we showed that the C-terminal cytosolic tail of Ste2 is necessary and sufficient for tethering Sst2 in vivo and that this interaction is disrupted by the Q304N mutation. Finally, we found that Sst2-Ste2 interaction is controlled by phosphorylation; Sst2 binds to the unphosphorylated tail of the receptor and cannot bind when the tail has undergone pheromone-induced phosphorylation.

In hindsight, it makes satisfying physiological sense that an RGS protein directly couples to the receptor that activates the G protein on which this regulator must act. One function of this DEP-domain-mediated anchoring to Ste2 is to ensure that the Sst2 RGS domain is situated in close proximity to its substrate, permitting immediate and efficient action (Figure 7). Thus, to ensure robust and sustained response to agonist, enough pheromone needs to be added to activate a sufficient number of receptors to outpace the amount of Sst2 initially present. Indeed, there is experimental evidence that basal Sst2 expression is adequate to squelch any Gα released by spontaneous G protein dissociation and sets the threshold of receptor activation required to trigger pheromone response (Siekhaus and Drubin, 2003). However, as mentioned earlier, Sst2 expression is highly induced by pheromone (Dohlman et al., 1996), providing a negative feedback loop to impose efficient downregulation after the initial response (Figure 7).

Based on much evidence, GPCRs associate primarily via their third intracellular loop with the Gα subunit of the coupled G protein, and Ste2-Gpa1 interaction is no exception to this general rule (Konopka and Thorner, 2004). However, there is genetic evidence that the cytosolic tail of Ste2 also interacts with Gpa1 in a "preactivation complex" (Figure 7), which contributes to preventing spurious agonist-independent G protein activation (Dosil et al., 2000). Consistent with this view, a dominant-negative mutant, Gpa1(N388D), appears to exert its effects via tighter binding to Ste2 (Wu et al., 2004) and may thus form a preactivation complex more stable than normal Gpa1. However, the wild-type interaction must be of relatively low affinity and must not occlude the Sst2 binding site because we clearly showed that Sst2 binds well to the receptor tail even when pheromone is absent. In agreement with the conclusion that Gα and Sst2 binding to the receptor are not mutually exclusive, addition of a 10- to 20-fold molar excess of Sst2 in pull-down assays does not detectably diminish the amount of Ste2 that binds to immobilized Gpa1(G302S) (unpublished data).

The fact that DEP-domain-mediated docking of Sst2 to the cytosolic tail of Ste2 is required for this negative regulator to carry out its function explains our prior observations that deletion of the Ste2 tail causes an increase (~300-fold) in pheromone sensitivity essentially equivalent to that caused in wild-type cells by an *sst2*Δ mutation and that *sst2*Δ cells expressing Ste2(Δ296) are only slightly more

and examined as in (A). An exponentially growing culture of a Gpa1(G302S) mutant coexpressing Sst2-GFP and a derivative of Ste2(7K-to-R)-mCherry that lacks 19 phosphorylation sites in the sequence from Ser331 to Thr425 (lower panels) was treated with 500 nM α factor for 1 hr and examined as in (A). Representative cells are shown in all panels.

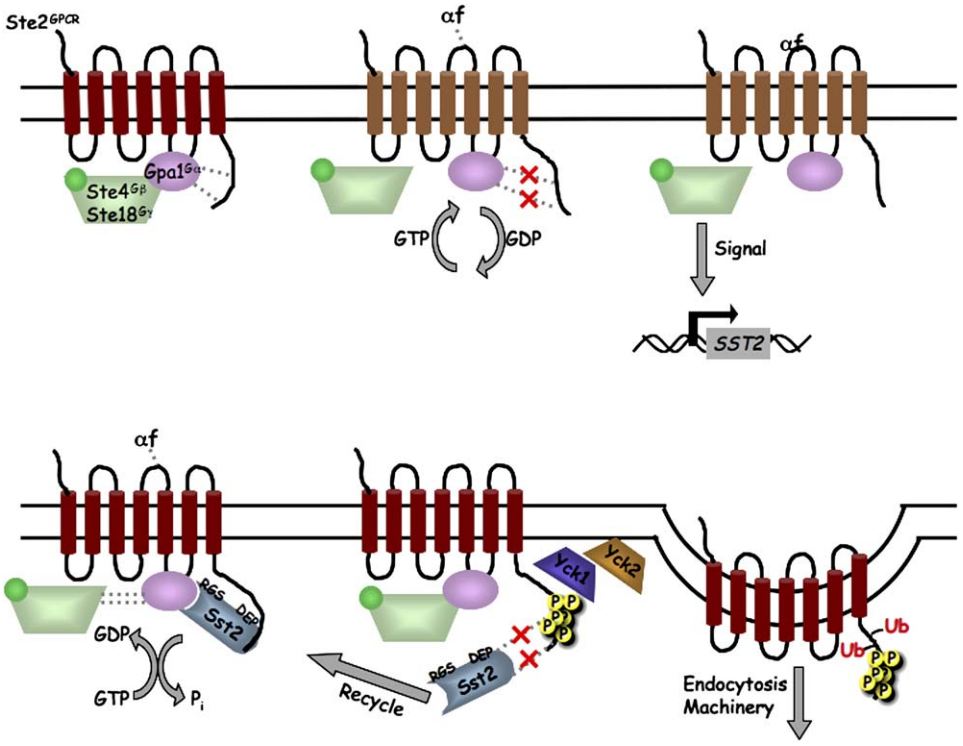

Figure 7. Model for DEP-Domain Function during Sst2-Mediated Desensitization
Dotted lines depict transition states; crosses mark disrupted interactions. See Discussion for further details.

pheromone sensitive ($\leq$10-fold) than either single mutant alone (Reneke et al., 1988). Perhaps the slightly additive effect represents elimination of the role that interaction of the cytosolic tail with Gpa1 plays in stabilization of the preactivation complex (Figure 7). One prediction of our findings is that Ste2(Δ296) should be more hypersensitive to pheromone action than any other alteration of the tail that we generated in this study, and this is the case (Figure S5).

Upon exposure to pheromone, we presume that agonist-induced conformational changes in the receptor relieve the constraints imposed by the preactivation complex and simultaneously facilitate interactions required for the receptor to exert its GEF activity on its associated G protein, releasing GTP-Gpa1 and the Gβγ complex (Figure 7). When the number of occupied receptors exceeds the amount of Sst2 present to reverse G protein activation, released Gβγ (which in yeast is the effector necessary to trigger downstream events [Dohlman and Thorner, 2001]) elicits pathway response, including induction of *SST2* and other pheromone-responsive genes (Figure 7). As the intracellular Sst2 concentration increases, it will be recruited to more and more receptors, where docking via its DEP domains positions its RGS domain to act optimally on any GTP-Gpa1 still being produced, thereby contributing to reformation of inactive G protein heterotrimers and downregulation of signaling. The same pheromone-induced conformational changes in Ste2 that establish its signaling-competent state also expose its cytosolic tail to phosphorylation by Yck1 and Yck2, which are plasma membrane-tethered by C-terminal S-palmitoylation (Feng and Davis, 2000). Phosphorylation concomitantly prevents Sst2 binding and serves as the signal to recruit the ubiquitin ligase that further modifies the receptor to trigger endocytosis (Hicke et al., 1998). This mechanism recycles Sst2, avoiding nonproductive interaction with receptors already en route to destruction (Figure 7).

Here we have showed that DEP domains in the RGS protein Sst2 are responsible for its specific recognition of a GPCR, Ste2. Direct association of an RGS protein with its cognate receptor provides a biologically sensible mechanism for achieving highly selective desensitization of any given GPCR-initiated pathway. It is likely, therefore, that other members of the RGS protein family, including worm EGL-10 and mammalian RGS6, RGS7, RGS9, and RGS11, which are expressed in the nervous system, associate with their cognate GPCRs (e.g., opioid and dopamine receptors) via DEP-domain-mediated contacts. Also, we suspect that DEP domains in fly Dsh and its mammalian Dvl orthologs associate with the GPCR-like receptor Frizzled in the Wnt signaling pathway and may be part of the switch that directs signals to the canonical Wnt-initiated β-catenin pathway or the planar cell polarity/convergent extension pathway, both of which require Dsh/Dvl (Huang and Klein, 2004). Likewise, DEP domains in the Rac GEF P-Rex-1 may mediate its direct association with the C5a receptor, a GPCR in neutrophils, which

stimulates the Gβγ-dependent activation of this exchange factor. Similar rationales can be invoked to explain why it would be physiologically reasonable for other types of DEP-domain-containing signaling proteins to specifically dock onto the GPCRs that initiate response in the pathways in which those proteins participate. In fact, sequence divergence among DEP domains may reflect the role they play in directing regulators and downstream effectors to the large array of GPCRs, which in metazoans are the single largest superfamily of gene products known. In the absence of a DEP domain, downstream components in GPCR-initiated pathways may have evolved other means to interact directly with their corresponding GPCRs. For example, RGS12 binds via a PDZ domain to the C terminus of CXCR2 (interleukin-8 receptor) (Snow et al., 1998), and RGS2 associates with the third intracellular loop of selected GPCRs (Hague et al., 2005). Alternatively, juxtaposition to cognate GPCRs can be mediated by contacts to other integral membrane proteins—for example, RGS7 interacts with the SNARE-like protein R7BP (Drenan et al., 2005; Martemyanov et al., 2003).

Although the DEP-domain-mediated interaction of Sst2 with the cytosolic tail of Ste2 places this RGS protein in close proximity to the Gα subunit it downregulates, it may serve other functions. One possibility is that Sst2 binding to Ste2 might also contribute to desensitization by sterically occluding G protein reassociation with the receptor, but, as mentioned above, our biochemical experiments make this an unlikely scenario. However, it is possible that binding of Sst2 contributes to downmodulation of signaling by inhibiting the ability of Ste2 to act as a pheromone-activated GEF. Our in vitro experiments in this regard have so far been inconclusive. In any event, DEP-domain-mediated interaction of Sst2 with the cytosolic tail of Ste2 achieves selective targeting of this regulator to its site of action and to its specific substrate, thereby ensuing that the GAP activity of its RGS domain acts efficaciously.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions

Cultivation of strains (Table S1) was at 30°C (unless otherwise indicated) in standard rich (YP) or defined minimal (SC) media containing 2% glucose (Glc), 2% raffinose with 0.2% sucrose (Raf/Suc), or 2% galactose (Gal) as required and supplemented with appropriate nutrients to maintain selection for plasmids where necessary. Standard yeast genetic techniques were used.

Plasmids and Recombinant DNA Methods

Plasmids (Table S2) were constructed and propagated in *Escherichia coli*, and site-directed mutagenesis using appropriate mismatch oligonucleotide primers was conducted, using standard recombinant DNA methods. Fidelity of all constructs was verified by nucleotide sequence analysis. Standard polymerase chain reactions (PCR) utilized Turbo *Pfu* DNA polymerase (Stratagene, La Jolla, CA, USA).

Assay of Sst2 Function

Sst2 action in vivo was assessed by its ability to reverse pheromone-induced growth inhibition using an agar diffusion (halo) bioassay (Reneke et al., 1988) in which filters were spotted with 15 μl of either a 0.1 mg/ml (59.3 μM) or 1 mg/ml stock (593 μM) of α factor or by its ability to squelch induction of a plasmid-borne pheromone-responsive reporter gene, pRS423-*FUS1*^{prom}-*lacZ* (Hoffman et al., 2002).

Selection for Loss-of-Function Alleles in the DEP Domains of Sst2

We exploited the fact that *sst2*Δ mutations elevate basal signaling in the pheromone response pathway (Dohlman et al., 1996; Siekhaus and Drubin, 2003). Hence, we used a *his3* strain carrying a pheromone-inducible *FUS1*^{prom}-*HIS3* reporter (Horecka and Sprague, 2000) and a low concentration (0.9 mM) of a competitive inhibitor of His3 (3-aminotriazole; 3-AT), which permitted growth of *sst2*Δ, but not *SST2*$^{+}$, cells. To avoid the pheromone-induced cell-cycle block, we included a specific allele, *far1(T306A)* (Gartner et al., 1998), that prevents Far1-imposed cell-cycle arrest but does not disrupt gene induction (Figure S2). See Supplemental Data for further details.

Selection for Dosage Suppressors of Sst2(Q304N)

An *sst2*Δ strain expressing myc-Sst2(Q304N) from the native *SST2* promoter on *CEN* plasmid was transformed with an *S. cerevisiae* genomic DNA library carried by a multicopy (2 μm DNA) plasmid, and the cells were plated on a concentration of pheromone (0.5 nM) that we determined empirically arrests the growth of *sst2*Δ cells expressing Sst2(Q304N) from the *SST2* promoter on a *CEN* plasmid for several days yet permits growth of the same cells in the same time period when Sst2(Q304N) is expressed from a multicopy (2 μm DNA) plasmid. Candidate library plasmids were rescued and retested, and only those that allowed growth of recipient cells dependent on the presence of Sst2(Q304N), but not in its absence, were further characterized. See Supplemental Data for details.

Interaction Assays

Myc-tagged Sst2 was overexpressed in protease-deficient yeast cells lacking endogenous Sst2, receptor, and G protein; stripped from the membrane fraction of lysates of these cells with high salt; and immunoaffinity purified. To prepare receptor, the plasma membrane fraction of lysates of *sst2*Δ cells expressing Ste2 at its endogenous level or lacking the receptor (*ste2*Δ) were purified by flotation through a sucrose gradient, solubilized with a mild nonionic detergent, and used immediately to assay binding to immobilized myc-Sst2. His_6-Sst2 was produced in insect cells using baculovirus expression, purified by FPLC, and used for binding to 3× FLAG-tagged Ste2 that was expressed in *sst2*Δ *ste2*Δ *gpa1*Δ *ste4*Δ yeast cells, solubilized with detergent, and immobilized on beads with anti-FLAG epitope mAb. The split-ubiquitin two-hybrid assay was conducting following established protocols (Fetchko and Stagljar, 2004). See Supplemental Data for details.

Fluorescence Microscopy

Cells affixed to microscope slides coated with 0.1 mg/ml concanavalin A (Sigma Chemical Co., St. Louis) in 10 mM MES (pH 6.0), 1 mM $CaCl_2$ were viewed under a 100× objective in a DeltaVision Spectris DV4 deconvolution microscope (Applied Precision LCC, Issaquah, WA, USA). Images were collected and processed using SoftWoRx imaging software (Applied Precision) and Photoshop (Adobe Systems, Inc., San Jose, CA, USA).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, two tables, and five figures and can be found with this article online at http://www.cell.com/cgi/content/full/126/6/1079/DC1/.

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